Isolation and Screening of Glutaminase & Urease Free Novel Fungal Strains for the Production of L-Asparaginase

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DECLARATION

I declare that this written submission represents my ideas in my own words and where ever others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the Institute and can also evoke penal action from the sources that have thus not been properly cited, or from whom proper permission has not been taken when needed.

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APPROVAL SHEET

This thesis entitled 'Isolation and screening of fungal strains for the production of Glutaminase and Urease free L-Asparaginase' by Nimmy Jose is approved for the degree of Master of Technology from Indian Institute of Technology Hyderabad.

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ABSTRACT

L-Asparaginase is an amidohydrolase that catalyzes the hydrolysis of amino acid L-asparagine into aspartic acid and ammonia. It is used in the treatment of Acute Lymphoblastic Leukemia (ALL) and some other malignant lymphoid abnormalities. It is also used in food industry to prevent the formation of acrylamide, a carcinogenic substance in carbohydrate rich fried and baked foods. Naturally L-Asparaginase is present in plants, animals and microbes but microorganisms such as bacteria, yeast and fungi are generally used for the production of L-Asparaginase as it is difficult to obtain the same from plants and animals. It is found that the L-Asparaginase from bacteria causes side effects in humans including anaphylaxis and serious allergic reactions which can be fatal in some cases. To overcome this, eukaryotic organisms such as fungi can be used for the production of L-Asparaginase. But sometimes the fungi produces L-glutaminase and urease enzymes along with L-Asparaginase which is difficult to remove in the purification stage. In order to prevent this fungal strains which can produce L-Asparaginase free of L-glutaminase and urease are isolated from different sources using standard protocols.

In the current study four novel fungal strains (C3-*Aspergillus sps*, C7-*Aspergillus sps*, W3-*Rhizopus sps*, W5-*Rhizopus sps*) producing L-Asparaginase free of L-glutaminase and urease are screened from a total of 40 fungal *sps* isolated from various soil samples and agricultural substrates collected from different locations. Activity studies are conducted for all these species according to standard protocols. Fungus with high enzyme index (C7) 1.57 was then subjected to Solid State Fermentation (SSF) studies in flasks and the results were compared with that of flask level Submerged Fermentation (SmF). The strain C7 is

found to have the highest activity of 44.09 U/ml in SmF and 22.41 U/ml in SSF at 72 hour of incubation at 35 ° C and 180 rpm.

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ABBREVIATIONS AND NOTATIONS

ALL	Acute lymphoblastic leukemia
BSA	Bovine serum albumin
BTB	Bromothymol blue
IU	International unit
MCDM	Modified Czapek Dox Medium
MTCC	Microbial Type Culture Collection and Gene Bank
OD	Optical density
PDA	Potato dextrose agar
PR	Phenol red
SmF	Submerged fermentation
SSF	Solid state fermentation
TCA	Trichloro acetic acid

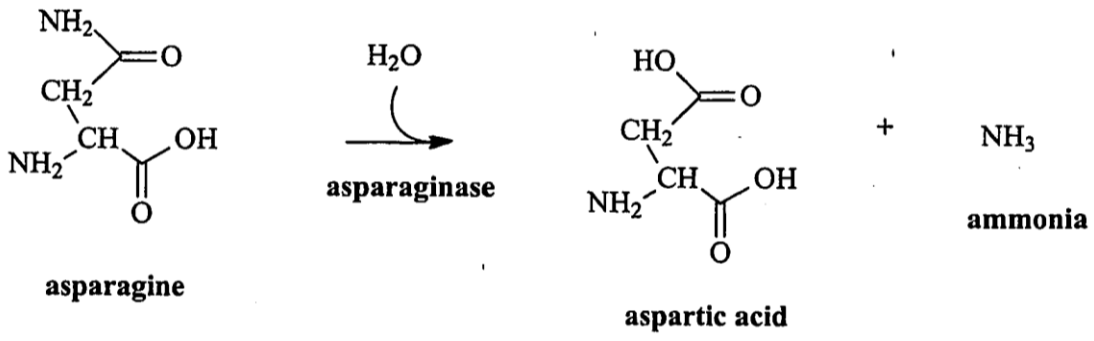
Chapter 1

INTRODUCTION

1.1 Generalities

L-Asparaginase (E.C. 3.5.1.1) is an enzyme which is found in a wide range of organisms including plants, microbes, animals and in the serum of certain rodents but not in human beings. It is an amidohydrolase, which catalyzes the hydrolysis of the amide group on the side chain of asparagine, an amino acid into aspartic acid and ammonia. It was first found to be present in the serum of guinea pigs by J G Kidd in 1953. He observed that the enzyme has tumor inhibitory properties and showed that transplanted lymphomas of mice and rat are repressed *in vivo* by repeated injections of guinea pig serum [1]. Because of its anti-tumor activities L-Asparaginase is used mainly in the treatment of Acute Lymphoblastic Leukemia (ALL). It is also used in the food industry to prevent the formation of acrylamide in fried food items [2]. L-Asparaginase is present in plants and mammals, since the extraction is difficult microbial sources especially bacteria and fungi are evaluated as potential source of enzyme production [3].

Figure 1. Hydrolysis reaction of L-Asparaginase on asparagine



1.2 L-Asparaginase in tumor treatment and its mechanism

Acute lymphoblastic leukemia (ALL) which mostly affects children is a form of cancer in which the bone marrow produces too many immature lymphocytes leading to reduced immunity. L-Asparaginase enzyme is used as a chemotherapy drug for the treatment of ALL. It is also used in the treatment of a number of lymphocytic cancers including Hodgkin's disease, non-Hodgkin's lymphoma, melanosarcoma etc. Normal cells can synthesize L-asparagine by itself because of the presence of the enzyme asparagine synthetase, whereas certain sensitive malignant cells cannot synthesize it by itself and require an external source of L-asparagine for optimal growth. During the treatment of ALL with L-Asparaginase, all the circulating asparagine in the body of the patient get hydrolyzed to aspartic acid and ammonia preventing the absorption of asparagine by tumor cells and hence depriving the dependent tumor cells of their extracellular source of L-asparagine. The asparagine deficiency rapidly impairs the protein synthesis and leads to delay in DNA and RNA synthesis and hence impairs the cell functioning finally resulting in cell death [4, 5]. L-Asparaginase is commonly used as a combination chemotherapy drug for the treatment of acute lymphoblastic leukemia (ALL) in children.

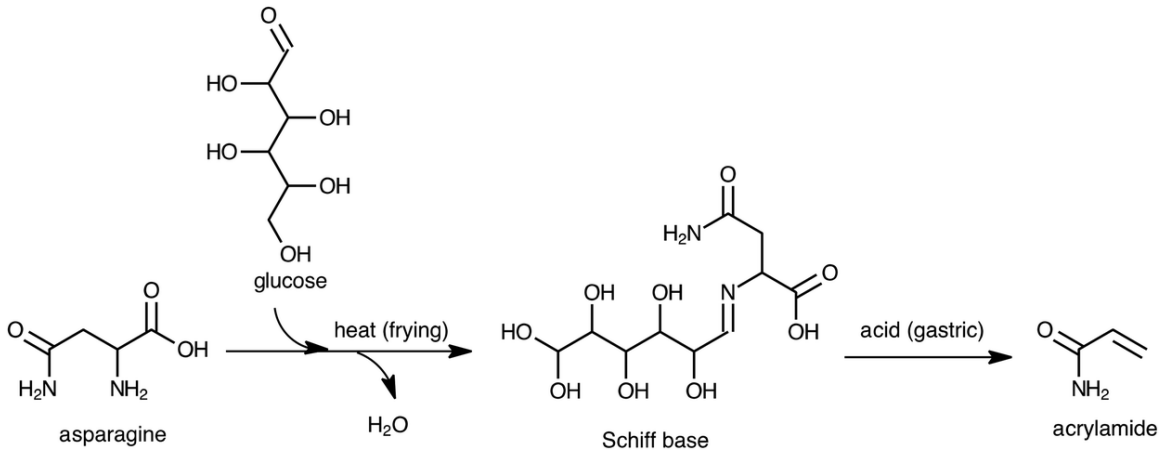
Unfortunately, despite the wide use of L-Asparaginase, most of the treatments have been interrupted due to severe side effects and immunological reactions in the patients. The side effects include anaphylaxis, coagulation abnormality, thrombosis, liver dysfunction, pancreatitis, hyperglycemia, cerebral dysfunction etc. These side effects are developed either due to the production of anti-asparaginase antibody in the body or due to multiple enzymatic activity of the produced enzyme [6]. Toxicity of L-Asparaginase is mainly due to the fact that the enzyme preparations are amidohydrolase, not L-Asparaginase. L-

Glutaminase and urease are usually associated with the L-Asparaginase isolated from most of the bacteria and fungi and it is very difficult to separate them in the purification stage [7]. These enzymes hydrolyze L-glutamine and urea in the body, thereby preventing kidney, central nervous system and other vital organs from normal functioning thus leading to serious side effects [8, 9].

1.3 Applications in food industry

This Enzyme is also used in the food industry to prevent the formation of acrylamide, a carcinogenic substance during frying or baking of food items containing starch at high temperatures [10]. The reaction is a result of heat induced Maillard reaction (or non-enzymatic browning reaction) between amino acid group of asparagine and carbonyl group of reducing sugar which provides desirable flavor to the food. On addition of the enzyme the asparagine in the food gets converted to aspartic acid and ammonia hence preventing the formation of acrylamide.

Figure 2. Maillard reaction of asparagine and glucose leading to the formation of acrylamide



L-Asparaginase production throughout the world is carried out either by submerged fermentation (SmF) or solid state fermentation (SSF). SSF is defined as the growth of microorganism on solid substrate which acts as an energy source in the absence of free flowing water. SSF is a substitute to submerged fermentation for the large scale production of industrial enzymes. The solid substrates used in SSF are mainly agricultural or industrial wastes which are cheap and has resistance to contamination especially for the large scale production of fungal enzymes. Therefore SSF can be used as a better method for the large scale production of L-Asparaginase.

1.4 Methods of production: Comparison of SSF and SmF

L-Asparaginase production throughout the world is carried out either by submerged fermentation (SmF) or solid state fermentation (SSF). Submerged fermentation is a process in which the growth of microorganisms takes place in liquid broth medium which is optimized with required nutrients to have a better cultivation of micro-organisms. This involves growing carefully the selected microorganisms in closed reactor containing the fermentation medium and a high concentration of oxygen. Submerged fermentation has well established equipment that make use of the existing micro-organisms. Bacteria is commonly used as source in this process as it requires high moisture content.

SSF is defined as the growth of microorganism on solid substrate which acts as an energy source in the absence of free flowing water [11]. SSF is a substitute to submerged fermentation for the large scale production of industrial enzymes. The solid substrates used in SSF are mainly agricultural or industrial wastes which are cheap and has resistance to contamination especially for the large scale production of fungal enzymes.

Compared to submerged fermentation SSF has many advantages, among those the most important thing is that it provides high yield and activity of the enzyme and the process is eco-friendly because it makes use of agricultural waste as the substrate and since the moisture content is low it avoids the need to treat a huge amount of effluent water. These factors avoid environmental pollution to a considerable extent.

SSF has disadvantages as well. The heat produced in SSF reactor is difficult to dissipate effectively hence it often leads to heat buildup which affects the growth of the fungi. The solid mass prevents effective diffusion of oxygen and the controlling of process parameters are really difficult.

Table 1. Comparison of SSF and SmF for enzyme production

Advantages		**Limitations**	
Submerged Fermentation	**Solid state fermentation**	**Submerged Fermentation**	**Solid state fermentation**
Better heat and mass transfer can be achieved	Low water requirement, resistance to contamination	Complex in operation, Low yield.	Heat build up Difficulties to ensure proper oxygen diffusion
Better diffusion of microorganism	No effluent water	High energy consumption and cost intensive	Large scale inoculums and difficult to control process parameters
Better diffusion of oxygen	Substrate are agricultural wastes		
Commercially available in large scale	High yield and product activity	High release of effluents	Difficulties in scale-up

1. 5 Objectives and scopes

Based on an extensive literature survey on the production of L-Asparaginase and characterization, the present study focused on isolation of a novel fungal strain for the production of glutaminase and urease free L-Asparaginase. The following objectives have been envisaged in the present investigation:

- Isolation and screening of potential glutaminase and urease free L-Asparaginase producing fungal strains from soil and agricultural samples.
- Identification of the strain with the maximum enzyme index.
- Comparison of activity studies in SmF and SSF.

These four strains C3, C7, W3 and W5 are free of glutaminase and urease and are found to have good L-Asparaginase activity and hence have high potential in the treatment of ALL. This is the first report on L-Asparaginase producing strain free of glutaminase and urease elsewhere reported in the literature.

1.6 Organization of thesis

The presentation of the work has been divided into five chapters. The current **Chapter 1** presents a general introduction, objective and scope of the present work. While the literature that supports the work is presented in **Chapter 2. Chapter 3** includes the details of the materials and methods adopted in the present study. It explains the procedures and protocols used in the study. **Chapter 4** contains the results and discussions. This chapter discusses in detail about the four isolates which are free of glutaminase and urease activity

and its SmF and SSF activity studies. **Chapter 5** draws summary and appropriate conclusions based on the previous results and discussions. It also provides some useful recommendations to carry out further work in this field.

Chapter 2
LITERATURE REVIEW

2.1 L-Asparaginase

L-Asparaginase (L-Asparaginase amidohydrolase EC 3.5.1.1) is the enzyme having antitumor activity and obtained from various biological sources *viz*., plants, animals and many other microorganisms (fungus, yeast, bacteria etc.). The enzyme acquired clinical importance in 1961 when the antitumor effect of Guinea pig serum originally discovered by Kidd. It has been used in leukemia treatment last four decades. The most common therapeutic indications are treatment of Hodgkin disease, acute lymphocytic leukemia (mainly in children), acute myelocytic leukemia, acute myelomonocytic leukemia, and chronic lymphocytic leukemia, lymphosarcoma treatment, reticle sarcoma and melanosarcoma. Recently, some more applications of L-Asparaginase have been reported in acrylamide free food production.

2.2 Historical development

The pioneer observation that turned out to be important for the development of L-Asparaginase as a potential antineoplastic agent was made by Clementi in 1922 revealing the presence of high activity of L-Asparaginase in the serum of guinea pig. High L-Asparaginase activity was observed only in guinea pig serum, whereas other mammals were found devoid of this enzyme [12]. Later in 1953 J G Kidd showed that transplanted lymphomas of mice and rat are repressed *in vivo* by repeated injections of guinea pig serum and found that some active constituent in serum is responsible for the selective necrosis of lymphoma cells [13]. The studies took another turn when Neumann and McCoy has

observed in 1956 that the basically non-essential amino acid asparagine is needed to grow the Walker carcinosarcoma 256 *in vitro* [14]. Haley and co-workers have found that murine L5178Y leukemia cells also require asparagine for *in vitro* growth in 1961 [15]. Broome also observed the same results in 1961 with his experiments with 6C3HED cell lines [16]. It was Broome who later in 1963 came up with the theory that the antitumor activities of guinea pig serum is due to the presence of the enzyme L-Asparaginase in it [17]. Looking at the biochemical reactions involved in these experiments it became evident that certain leukemic blast cells are sometimes unable to synthesize enough asparagine for their own metabolism, so that the asparaginase-induced deficiency in asparagine will impair cellular function and eventually cause cellular death. So the specificity of L-Asparaginase towards L-asparagine is the reason behind this therapeutic effect.

Furthermore, a major advancement resulted when Mashburn and Wriston in 1963 reported that asparaginase can be extracted from *E.coli* bacteria and it can inhibit the growth of tumor cell just like guinea pig serum [18].This opened the possibilities to produce and utilize the enzyme in larger quantities. It also leads to number of clinical studies [19]. The first clinical trials in patients with acute lymphoblastic leukemia were carried out with asparaginase preparations both from guinea pig serum and *E. coli*. Both enzymes showed clinical efficacy [20].

In the later years further studies identified more bacterial species with L-Asparaginase producing capability. Among those isolates *Erwinia Chrisanthemi* showed maximum activity and it was used for large scale production of the enzyme [21]. Even though a large number of strains were reported to have L-Asparaginase activity in the following years

including only *E Coli* and *Erwinia Chrisanthemi* species were widely used for large scale production.

Treatment with *E Coli* protein was always found to be associated with hypersensitivity reactions. Whereas the *Erwinia Chrisanthemi* protein was found to have negligible or lesser side effects in clinical trials [22]. But both the protein have certain level of immunogenicity. Later it was found that coupling the derived protein with Poly Ethylene Glycol (PEG) group could preserve the activity of the enzyme for a longer time and could reduce the immunogenicity to certain extend [23]. It helped to reduce the hypersensitivity of the enzyme and allowed much less frequent administration of PEG-asparaginase compared to normal asparaginase.

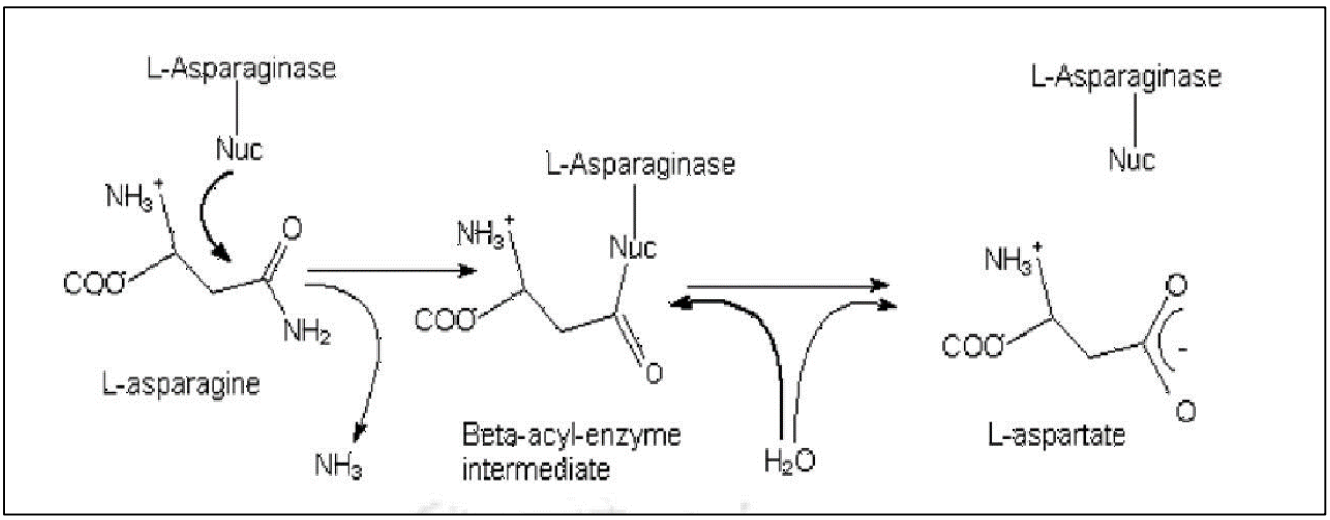
2.3 Chemistry and structural aspects of L-Asparaginase

Enzymes with L-Asparaginase activity can be generally classified into two groups, the bacterial-type and the plant-type L-Asparaginases, characterized by different structural and biochemical features. The bacterial-type enzymes are further grouped into type I and type II depending on their cellular localization and substrate specificity. Type I includes cytosolic enzymes that exhibit low affinity for L-Asparaginase, whereas type II enzymes are localized in the periplasm and show considerably higher affinity for L-Asparaginase [24]. These enzymes from various sources have been purified and its biochemical properties are studied extensively over the last 4 decades. Type II asparaginase has a stable tetrameric structure composed of 4 identical sub units and each subunit contains 326 amino acid residues [25]. *E.Coli* asparaginase has molecular weight of approximately 130 kDa and the affinity constants for L-asparagine and L-glutamine are 1.15×10^{-5} and 6.25×10^{-3} M, respectively. The isoelectric point of crystalline type varies from 4.8 to 5.6 [26, 27].

Whereas the molecular weight of *Erwinia* L-Asparaginase is between 135 - 138 kDa and specific activity of the purified enzyme lies between 300 and 400 mole of the substrate per minute per milligram of protein. The isoelectric point ranges between pH 4.6 and 5.5 for E. coli enzyme, and is around 8.7 for the *Erwinia* enzyme [28].

The amidohydrolase L-Asparaginase helps in the hydrolysis of non-essential amino acid asparagine into aspartic acid and ammonia. L-asparagine hydrolysis is known to proceed in two steps. In the first step a covalent intermediate, beta-acyl-enzyme intermediate is formed through nucleophilic attack by the threonine group on L-Asparaginase as shown in figure 3. In the second step, a water molecule attacks the acyl-enzyme intermediate to produce L-aspartate and ammonia [29]. The structure of *E. coli* L-Asparaginase was studied by Swain *et al.*, (1993) and two domains were observed [25]. Location of the active site was found to be between the N and C terminals. Structure of the enzyme with bound L-aspartate indicated a threonine residue as a catalytic nucleophile by Miller *et al.* in 1993 [30]. Hydrolysis reaction is assayed by measuring the release of ammonia using Nessler's reagent or by measuring the release of L-aspartate.

Figure 3. Schematic illustration of the reaction mechanism of L-Asparaginases. The proposed covalent intermediate is formed through nucleophilic attack by the enzyme. Bold arrows indicate nucleophilic attack



2.4 Sources of L-Asparaginase

Microorganisms are considered as effective sources for the production of therapeutic enzymes since microbes are easy to manipulate. Broad range of microorganisms such as filamentous fungi, yeast, actinomycetes and marine organisms are isolated from different sources.

2.4.1 Bacterial L-Asparaginase

L-Asparaginase production from various bacterial sources have been studied extensively over decades due to the flexibility with which bacteria's can be manipulated. L-Asparaginase from *E.coli* and *Erwinia chrysanthemi* are clinically used for the treatment of ALL. Bacterial asparaginase derived from various bacteria differ in pH, molecular weight, stability and affinity and they are serologically and biochemically different even though the toxicity, anti-neoplasticity and immunogenicity are similar. Bacterial formulations are found to have high immunogenicity in ALL treatment. Different bacterial isolates with L-Asparaginase activity reported in the literature are given in table 2.

2.4.2 Fungal L-Asparaginase

Bacterial L-Asparaginase is often associated with hypersensitive reactions in patients which can be fatal in some cases. This leads to the studies to identify fungal strains which are free of allergic and immunogenic reactions. Since the fungi are eukaryotic organisms and evolutionarily more close to human cell line the immunogenic side reactions are comparatively lesser for fungal asparaginase. The mitosporic fungi genera such as *Aspergillus*, *Penicillium* and *Fusarium* are commonly reported in the literature to produce asparaginase [31, 32, 33, 34]. Imada et al. observed that amidase activity is present in

fungal strains, *Penicillium clavgorme* and *P. expansum*. Sarquis *et al.*, (2004) and Mishra, (2006) reported that the L-Asparaginase production by *A. terreus* and *A. niger*, respectively [8, 35]. Other isolates are given in the table.

2.4.3 Actinomycetes L-Asparaginase

Recently other than terrestrial based microorganisms focus has been shifted to marine microbes for the production of bioactive compounds. Marine biosphere is a potential source of actinomycetes from which various antibiotics and bioactive compounds can be derived. L-Asparaginase from marine actinomycetes showed cytotoxic effects on acute T cell leukemia and mylegeneous leukemia [36]. Dharmraj (2011) reported production of L-Asparaginase from marine actinomycetes, and purified enzyme showed a final specific activity of 78.88 IU/mg at pH 8 [37]. L-Asparaginase production from numerous actinomycetes such as *Streptomyces* ABR2, *Streptomyces albidoflavus* have been explored it is given in table 2.

Table 2. Various microbial sources of L-Asparaginase

Bacteria	**Reference**	**Fungi**	**Reference**
E. coli	[18]	*Aspergillus terreus*	[31]
Erwinia aroideae	[38]	*Fusarium tricinctum*	[41]
Serratia marcescens	[39]	*Aspergillus niger*	[35]
Erwinia carotovora	[40]	*Aspergillus terreus* MTCC 1782	[42]
Enterobacter aerogenes	[43]	*Fusarium equiseti*	[54]
Enterobacter aerogenes	[44]	*Penicillium sp.*	[55]
Thermus thermophilus	[45]	*Penicillium digitatum.*	[56]

Pseudomonas aeruginosa 50071	[46]
Erwinia carotovora	[47]
Bacillus circulans MTCC 8574	[48]
Serratia marcescens SB08	[49]
Bacillus sp.	[11]
Pectobacterium carotovorum MTCC 1428	[50]
Serratia marcescence	[51]
Bacillus licheniformis	[52]
Mycobacterium phlei	[53]
Thermus aquaticus.	[34]
Cladosporium sp.	[57]
Saccharomyces cerevisiae	[58]
Rhodosporidium toruloides	[59]

Actinomycetes	**Reference**
Streptomyces albidoflavus.	[60]
Marine actinomycetes	[61]
Streptomyces noursei MTCC 10469	[37]
Streptomyces ABR2	[62]
Nocardia sp.	[63]
Streptomyces longsporusflavus	[64]
Streptomyces gulbargensis	[65]

2.5 Clinical availability of L-Asparaginase

Current form of L-Asparaginase therapy involves injecting the drug preparation either intravenously or intramuscularly. Preparation from *E coli* and *Erwinia Chrisanthemi* asparaginases are clinically available in the market now along with peglylated form of *E coli* asparaginase. *E. coli* and *Erwinia* asparaginases have identical mechanisms of action but their kinetic properties are different, and patients sensitive to one drug have often show tolerance to the other. There are several different types of L-Asparaginase available commercially, each derived from a different bacterium. Patients receiving treatment with L-Asparaginase derived from *Escherichia coli* (*E. coli*), who develop hypersensitivity to

that form of the enzyme, may be able to continue treatment with Erwinase® as the enzymes are immunologically distinct. Immunologic cross-reaction between antibodies against various formulations of native L-Asparaginase from *E. coli* and PEG L-Asparaginase has been reported, but no such reaction has been found against *Erwinia* L-Asparaginase [66]. Antibodies targeting *E. coli* derived L-Asparaginase have been shown not to cross-react with Erwinase. *Eriwinia* asparaginase is recommended to patients having high allergic reactions [67]. To eliminate the high immunogenic reactions of *E Coli* Asparaginase the protein was conjugated with PEG group (PEG Asparaginase) which reduced the side effects to a greater extend. Available commercial forms of L-Asparaginase are listed in Table 3.

Table 3: Available commercial forms of L-Asparaginase

Source	**Marketed by**	**Trade name**
E.coli asparaginase	EUSA Pharma, Oxford, UK	Kidrolase
	Ovation Pharmaceuticals, Deerfield, Illinois	Elspar
	Bayer AG, Leverkusen, Germany	Crasnitin
	Sanofi-Aventis, Paris, France	Leunase
	Kyowa Hakko, Tokyo, Japan	Asparaginase Medac
	Rhône-Poulenc Rorer	Ciderolase
PEG-asparaginase	Sigma-Tau Pharmaceuticals, Inc. Gaithersburg, Maryland	Oncaspar
Eriwinia asparaginase	EUSA Pharma, UK	Erwinase
	Speywood	Erwinase

2.6 Treatment and side effects

Current treatment protocols of ALL and lymphosarcoma do not employ L-Asparaginase as a single agent it is always a part of multiple agent regimens and combined with drugs having definitive immunosuppressive effects. Acute lymphoblastic leukemia cell lines have been markedly inhibited by asparaginases. L-Asparaginase induces cell cycle arrest in G1 phases, which results in apoptosis of leukemia cells [68]. *E.coli* L-Asparaginase has been found to phosphorylate endogenous polypeptides in immune cells. Products of L-Asparaginase hydrolysis action especially $NH4^+$ ions diffuse into the cytosol of the tumor cell and modify the pH, which activates signal transduction pathways associated with phosphorylation of substrates [69]. It was reported that L-Asparaginase has been found to be effective in nasal type leukemia treatment [70]. It was found that the deamination of glutamine may enhance the anti-leukemic effect of L-Asparaginase [71]. Asparagine levels have been found to be strongly correlated with plasma L-Asparaginase activity, even at low enzyme activities of <50 U/l. Asparagine levels have an inverse relation with L-Asparaginase activity and chemotherapy. L-Asparaginase results in decreased asparagine, glutamine and 5 other amino acid levels in pediatric patients with ALL [72].

Besides minor side effects such as an allergic reaction and vomiting, L-Asparaginase therapy has some serious side effects which can be fatal in some cases. Onset of venous thrombosis in children undergoing histopathologic disease due to ALL therapy has been reported by Sahoo and Hart in 2003 [73]. Meyer *et al* has reported L-Asparaginase associated hyperlipidemia with hyper viscosity syndrome in a patient with T-cell lymphoblast lymphoma [74]. *Neutropenic enterocolitis* has been observed as an unusual acute complication of neutropenia, associated with leukemia and lymphoma.

Hypersensitivity reactions to chemotherapeutic antineoplastic agents such as L-Asparaginase have been reported. Tubular and glomerular dysfunction due to ALL chemotherapy was reported by Ikarashi *et al.,* (2004) [75]. Ching *et al.,* (2004) observed that the L-Asparaginase treatment causes urethral obstruction during treatment of ALL [76]. Myocardial ischemia has been observed in a patient with acute lymphoblastic leukemia during asparaginase treatment [77].

2.7 Large scale production of L-Asparaginase

The usage of this enzyme in the clinical field requires large scale production using microbes. Different modes of operation and different microbes are used for the large scale production. Each operation requires careful controlling of parameters such temperature, pH, dissolved oxygen content, agitation rate etc.

2.7.1 Production of L-Asparaginase by Submerged fermentation

Submerged fermentation is a process in which the growth of microorganisms takes place in liquid broth medium which is optimized with required nutrients to have a better cultivation of micro-organisms. This involves growing carefully the selected microorganisms in closed reactor containing the fermentation medium and a high concentration of oxygen. Submerged fermentation has well established equipment that make use of the existing micro-organisms. Bacteria is commonly used as source in this process as it requires high moisture content. Production of L-Asparaginase from various microbial sources using submerged fermentation is listed in Table 4. Fungal species such as *Aspergillus niger, A.terreus, A.tamari, Fusarium* and *Pencillium* are reported for L-Asparaginase production by submerged fermentation [78]. Studies on optimization of culture media and environment conditions in both batch and continuous SmF have been

reported. Synthesis of L-Asparaginase hinge on type of the organism and various parameters such as pH of medium, temperature, nutrient composition, inoculation concentration, dissolved oxygen concentration and fermentation time [79].

Table 4. Summary of fermentation conditions and microbial cultures for production of L-Asparaginase using SmF

Fermentation medium (w/v)	Microorganism	Fermentation Parameters	L-Asparaginase yield	Specific Activity	Reference
Autolyzed yeast extract	*Serratia marcescens* ATCC 60	Temperature 26 °C pH 5.0;	3.7 IU/mL		[80]
Tryptone 0.05; Glucose 0.1; Yeast 0.05	*Erwinia aroideae* NRRL B-138	Temperature 28 °C, pH 7.0	1,250 IU international unit /g dry weight of cells		[38]
Peptone 4.0	*Streptomyces griseus* ATCC 10137	pH-8.5; batch	117 IU/L		[81]
tryptone 0.5; yeast extract 0.3; NaCl 0.2; Glucose 0; $FeCl_3$ 2 µM; 1 mM $MgCl_2$; 0.2 mM $CaCl_2$	*Thermus thermophilus*	Temperature 70°C, pH 7.0	-	840 IU/mg protein.	[45]
proline medium 2	*Aspergillus terreus*	Temperature 30°C, pH 6.2	58.8 U/L		[8]
Asparagine dextrose salt medium	*Streptomyces griseus* NIOT-VKMA29	Temperature 35°C, pH 8.0	56.78 IU/mL		[82]

2.7.2 Production of L-Asparaginase by Solid state fermentation

L-Asparaginase is usually produced through submerged fermentation technique. But this method has several disadvantages such as low product yield, high cost and it generates large amount of waste water effluent. Solid state fermentation is extensively used in various processes such as bioremediation, bio detoxification of various hazardous compounds,

production of various therapeutic enzymes and secondary metabolites and as an effective alternative to submerged fermentation. Solid State Fermentation (SSF) is defined as the process in which the growth of micro-organism takes place on a solid substrate in the absence or presence of little water or with minimal moisture content. In this technique solid substrate is considered as the only source for the microorganisms. SSF use agricultural wastes and industrial residues that are cheap and readily available material as source of growth for microorganism for the production of low volume-high cost products. L-Asparaginase was produced from marine actinomycetes by SSF and SmF and a comparison showed that L-Asparaginase with higher activity is isolated from SSF process [83]. Substrates such as wheat bran, coconut oil cake, black sesame oil cake, soy bean meal, sugar cane bagasse are commonly used for the SSF production of the enzyme. Mishra (2006) testified maximum production of L-Asparaginase from *Aspergillus niger* using *Glycine max* as substrate followed by that of, *Phaseolus mungo and Cajanus cajan* [35]. Many statistical methods have been reported in optimization of process for the extraction of L-Asparaginase from microbes. Estimation of nutritional and environmental factors are essential for the production of enzyme. Statistically designed and planned experiments screen and evaluate the response of different parameters which enhances the enzyme production. L-Asparaginase production on wheat bran using *Cladosporium* sp. was studied with five experimental design variables pH, temperature, moisture content, inoculum concentration and time using response surface methodology with enzyme yield as 3.74 U [84]. L-Asparaginase production on sesame oil cake using *Aspergillus niger* C4 in a SSF column bioreactor was optimized for three variables (aeration rate, bed thickness and temperature) which resulted in maximum L-Asparaginase yield of 310 U/gds [85].

Table 5. Summary of microbial cultures and fermentation conditions for production of L-Asparaginase using SSF

Substrate	Microorganism	Fermentation Parameters	Enzyme Activity	Specific Activity	Reference
Soy bean meal	*Pseudomonas aeruginosa* 50071	Temperature 37 °C ; pH 7.4; moisture content 40% (w/v);	142.18 IU		[46]
Soy bean meal	*Pseudomonas aeruginosa* 50071	Temperature 37 °C ; pH 7.4; moisture content 50% (w/v);		1900 IU/mg	[86]
Glycine max	*Aspergillus niger*	Temperature 30 ± 2° C; pH 6.5; moisture content 70% (w/v)	40.9 ± 3.35 U/g of dry substrate		[35]
Red gram husk.	*B. circulans* (MTCC 8574)	Temperature 30 ° C; pH 7.0; moisture content 40% (w/v)	780 U/g of dry substrate		[48]
Bengal gram husk.			600 U/g of dry substrate		
Ground nut cake.			360 U/g of dry substrate		
Coconut oil cake.			380 U/g of dry substrate		
groundnut oil cake,	*Aspergillus terreus* MTCC 1782	Temperature 30 ° C; pH 6.2	30.35 IU/mL		[42]
Rice bran with nutrients	*Serratia marcescens* SB08	Temperature 30 ° C; pH 7.0; moisture content 50% (w/v)	79.84 U/g		[49]
Soya bean meal	*Fusarium equiseti*	initial moisture content 70%	8.51 IU		[54]
Wheat bran	*Cladosporium sp.*	moisture content of 58%, pH - 5.8, temperature 30°C	3.74 U		[84]
Coconut oil cake	*Serratia marcescens*	initial moisture content of substrate 50%, Temperature 35.5°C and pH - 7.4.	5.86 U/g of dry substrate		[51]

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

Chemicals and reagents used in the isolation and screening study were of analytical grade and obtained from Himedia and SRL. L-asparagine used in the assay is from Sigma Aldrich, India.

The agricultural substances used for the isolation and SSF studies were purchased from local market in Hyderabad.

3.2 Fungal species

MTCC 1782 *Aspergillus Terreus* was purchased from Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India. The strain was revived and maintained on potato dextrose slants. The organism was sub-cultured every month and stored at 4±1°C.

3.3 Collection of soil samples

Soil samples were collected from Western Ghats of and Kerala and samples were collected in air tight containers and kept at room temperature in laboratory.

3.4 Plate assay for the screening of L-Asparaginase production

Modified Czapek Dox medium(MCM) with composition glucose 2 g L^{-1}, L-asparagine 10 g L^{-1} , KH_2PO_4 1.52 g L^{-1}, KCl 0.52 g L^{-1}, $MgSO_4.7H_2O$ 0.52 g L^{-1}, $FeSO_4.7H_2O$ trace, $ZnSO_4$. $7H_2O$ trace and agar 18 g L^{-1} was prepared. 2.5 % (w/v) stock solution of the phenol red dye was prepared and MCM medium was supplemented with 0.009% phenol red dye. pH of this medium was adjusted to 6.2 using 1 M NaOH [87]. Prepared media was autoclaved and poured into pre-sterilized plates. Control plates were prepared with $NaNO_3$

as nitrogen source instead of L-Asparagine. Both MCM and control plates were inoculated with fungal strains and incubated for 96 h. The MCM plates will change the color from yellow to pink if the microbe is producing L-Asparaginase enzyme. The enzyme will hydrolyze the asparagine present in the media to ammonia and L-aspartate which increase the pH of the media which causes phenol red to change the color from yellow to pink. So a pink color change will be visible on the plate around the colony.

Mahajan et al developed a different MCM plate assay with Bromothymol blue as the dye instead of phenol red. 0.04% of stock solution of the Bromothymol blue dye was prepared and 0.007% BTB dye was supplemented in MCM medium. Final pH of the media was adjusted at 5.5 using 1M NaOH [88]. The BTB dye will change the color of the media from yellow to blue if the microbe shows asparaginase activity. 007% BTB is reported to have sharp color contrast zone, ranging from yellow at acidic pH, green at neutral pH to blue at alkaline pH.

3.5 Plate assay for L-Glutaminase production

L-glutaminase activity of the fungal strains are detected by supplementing the modified Czapek Dox medium with L-Glutamine as sole nitrogen source instead of L-asparagine. Strains were inoculated and observed for color change from yellow to pink in case of phenol red dye and yellow to blue for BTB dye.

3.6 Plate assay for Urease production

MCD medium without nitrogen source was autoclaved and 1% filter sterilized urea solution was added to MCD media for detection of urease producing fungi. This method is followed for producing MCD media with urease because otherwise urease decomposes at

high temperature during sterilization. Test strains were inoculated and observed for change in the color of the medium.

3.7 Isolation and screening of fungi from soil and agricultural samples

Fungal strains are isolated from 4 different soil samples from Western Ghats and 4 different agricultural substrates, cotton seed oil cake, wheat bran, rice husk and red gram husk by both serial dilution method and soil plate method. In serial dilution method 5 g of sample is mixed in 50 ml of .9% saline solution and incubated for 30 minutes in an incubator shaker. This is then serially diluted up to 10^{-6} dilution and inoculated on the Modified Czapek Dox agar plates incorporated with .009% phenol red dye with L-asparagine as a sole nitrogen source and incubated at 30°C for 4 days. In soil plate method 2 g sample is uniformly spread on the MCD agar plates and incubated for 4 days at 30°C [89]. Once fungal colonies are grown the plate shows pink coloration if the fungi is producing L-Asparaginase. Then those fungal strains were again screened separately in MCD plates and if found positive it is grown in potato dextrose slants and stored at 4^{o}C. For the reproducibility of results the isolated strains are again screened in liquid MCD medium.

3.8 Analytical methods

3.8.1 Assay of L-Asparaginase

L-Asparaginase activity is obtained by measuring the ammonia liberated when L-Asparaginase is allowed to react with L-asparagine. The liberated ammonia is then reacted with Nessler's regent which forms an orange precipitate. The optical density of this solution is measured in a spectrophotometer [Beckman Coulter] at 425 nm. A modified protocol of Writson (1970) [90] as described by Sanjay Kumar et al. is used for the assay [91]. A freshly prepared potato dextrose slant of the fungal strain was supplemented with

1 mL of autoclaved DD water to prepare the inoculum. 50 mL autoclaved MCD medium in a 250 mL Erlenmeyer flasks was inoculated with 2% of the inoculum [cell density: 6 X 10^8] and incubated in an orbital shaker incubator at 30^{o}C and 180 rpm. Samples were withdrawn at regular intervals to analyze for L-Asparaginase production.

The enzyme samples are filtered and the supernatant was analyzed for extracellular L-Asparaginase activity. The enzyme assay mixture consisted of 900 µL of freshly prepared L-asparagine (10 mM) in Tris–HCl buffer (pH 8.6) and 100 µL of filtered crude enzyme. The reaction mixture was incubated at 37 ^{o} C for 30 min and the reaction was stopped by adding 100 µL of 1.5M trichloroacetic acid (TCA). The reaction mixture was centrifuged at 10,000 rpm for 5 min at 4 ^{o} C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 100 µL Nessler's reagent into the sample containing 100 µL supernatant and 800 µL distilled water. The contents in the sample were vortex mixed and incubated at room temperature for 10 min and OD was measured at 425 nm against the blanks that received TCA before the addition of crude enzyme. The ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One unit (IU) of L-Asparaginase activity was defined as the amount of the enzyme that liberates 1 µM of ammonia/ min at 37 ^{o} C. Specific activity is expressed as units/mg of protein.

3.8.2 Protein estimation

The total protein content of the samples were determined according to the method described by Lowry *et al.,* (1951) [92]. The protein assay mixture consisted of 200 µL of crude extract of enzyme and 1mL of freshly prepared complex forming reagent (2%

Na_2CO_3 in 0.1 N NaOH: 1.0% $CuSO_4.5H_2O$: 2% potassium sodium tartrate ≡ 100:1:1). The contents in the sample were vortex mixed and incubated at room temperature for 15 min. Then 100 µL of freshly prepared diluted Folin phenol reagent with distilled water (1:1) was added and vortex mixed and kept in the dark at room temperature for 30 min. OD at 660 nm was measured against the blank (no crude enzyme, 200 µL distilled water). The protein concentration in the reaction was determined based on a standard curve obtained with bovine serum albumin as standard.

3.9 SSF studies

Solid state fermentation studies of the selected isolate W_5 (strain with the high enzyme index) was done in 250 mL Erlenmeyer flasks. 20 g of the sterilized agricultural substrate moistened to 60% of the dry weight with sodium phosphate buffer of pH 7 is taken in in the flask and inoculated with 1 mL inoculum prepared by adding 1 mL of autoclaved DD water in to a freshly prepared potato dextrose slant. It is then incubated at 35^o C for 96 h in an incubator and samples are collected and analyzed at equal intervals.

For the assay of L-Asparaginase enzyme produced in solid state fermentation 2g substrate is withdrawn at equal intervals of time and 10 mL (1:5) of pH 7 sodium phosphate buffer is added and it is kept in an incubator shaker at 30 ^{o}C and 180 rpm for one hour. After that the sample is filtered and filtered in Whatman no 1 filter paper. This enzyme extract is used for the assay as discussed in the previous section.

Chapter 4

RESULTS AND DISCUSSION

4.1 Isolation of fungal strains from different sources

A total of 30 different fungal isolates were obtained from the isolation studies conducted with 4 different soil samples and 4 different agricultural substrates. Out of which 20 isolates are found to have L-Asparaginase activity and can be used for the production of the potential enzyme. Out of these most of the fungi belonged to A*spergillus, Rhizopus* and *Pencillium* species.

Table 6. List of isolation sources and strains

Source	Region	Description	No. of isolates	Name of the isolates
Soil samples	Kerala	Soil samples were collected from Western Ghats	5	$S_{1.1}$,$S_{1.4}$ $S_{2.1}$, $S_{3.1}$, $S_{3.4}$
Agricultural residues	Substrates were collected from local market.	Cotton seed oil cake	6	C_1,C_3,C_4,C_5,C_6,C_7
		Rice husk	2	R_1,R_2
		Wheat bran	5	W_1,W_2,W_3,W_4,W_5
		Red gram husk	2	P_2,P_3

4.2 Screening studies of the isolated fungal *sps*

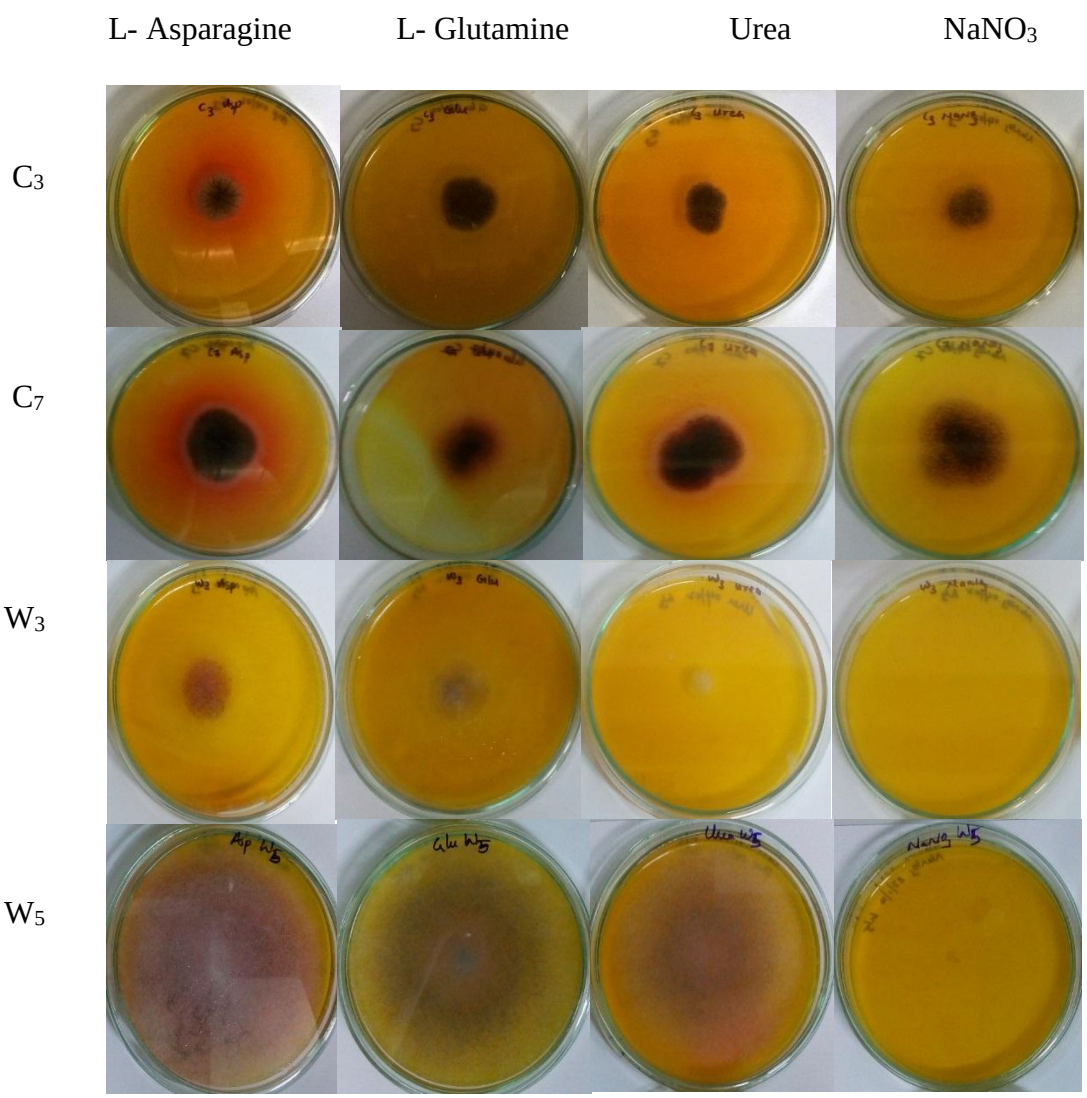
This study involved the screening of all the isolated fungi for the production of multiple enzymes. Most of the L-Asparaginase producing bacteria or fungi produce the enzyme along with L-glutaminase and urease enzymes which are not desired at all for the therapeutic applications as described in the previous paragraphs. So all the isolated fungi were screened for the production of L-Asparaginase, L-glutaminase and urease using modified Czapek Dox medium implemented with asparagine, glutamine and urea respectively as the sole nitrogen sources as described in materials and methods. Screening

studies were conducted with both phenol red and BTB dyes for the reproducibility of the results. These amidohydrolases, cleave amine groups and liberates aspartic acid and ammonia in case of L-Asparaginase, glutamic acid and ammonia in case of L-glutaminase and carbonic acid and ammonia in the case of urease. Ammonia liberated in the medium further reacts with water to produce NH_4OH resulting in increase in the pH of the medium which changes the color of the dye in the medium.

Phenol red dye is yellow at acidic pH and turns pink at alkaline pH, presence of pink color zone around the colonies on MCD plates with different nitrogen sources is due to the liberation of corresponding enzyme. BTB dye change the color from yellow at acidic pH to green ant neutral and blue at alkaline pH. Twenty isolates showed pink zone around the colonies indicating increase in pH. Fungal isolates W_3, W_5, C_3, and C_7 showed presence of pink color zone around the fungal colonies only on the asparagine plate indicating that these isolates produce only L-Asparaginase enzyme and no glutaminase or urease enzyme is produced. MTCC 1782 strain showed pink color zone when grown on L-asparagine, L-glutamine and urea plates indicating that strain produces 3 enzymes. To ensure reproducibility, all the isolates were screened with BTB as both the dyes are formulated for screening the hydrolysis of L-glutamine, L-asparagine and urea. Among phenol red and BTB, 0.007% of BTB dye showed sharp and clear color zone which makes it better than the phenol red dye. Methyl red was incorporated as pH indicator in the recent study to screen L-Asparaginase and L-glutaminase producing microorganism [93]. Out of twenty strains four show only asparaginase activity 4 show asparaginase and urease activity and 12 show asparaginase glutaminase and urease activity. 12 strains produce all the three enzymes i.e. 60% of the total isolated strains. This is the first study on isolation and

screening of glutaminase and urease free L-Asparaginase from fungi. Also, several fungi were identified that have potential of producing glutaminase free L-Asparaginase. Screening results of all the strains are indicated in table 7 along with colony diameter and zone diameter.

Figure 4. Screening of isolated strains for multiple enzyme activity using MCD plates amended with .009% phenol red



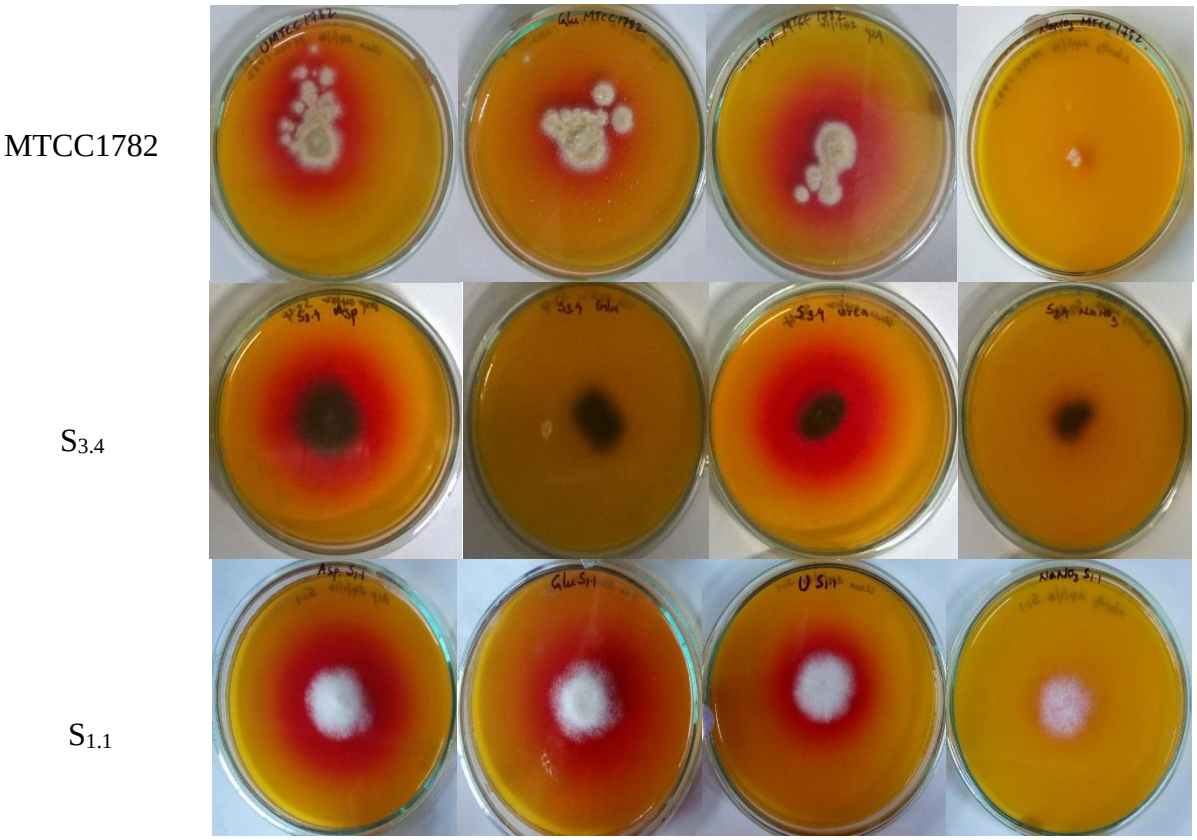
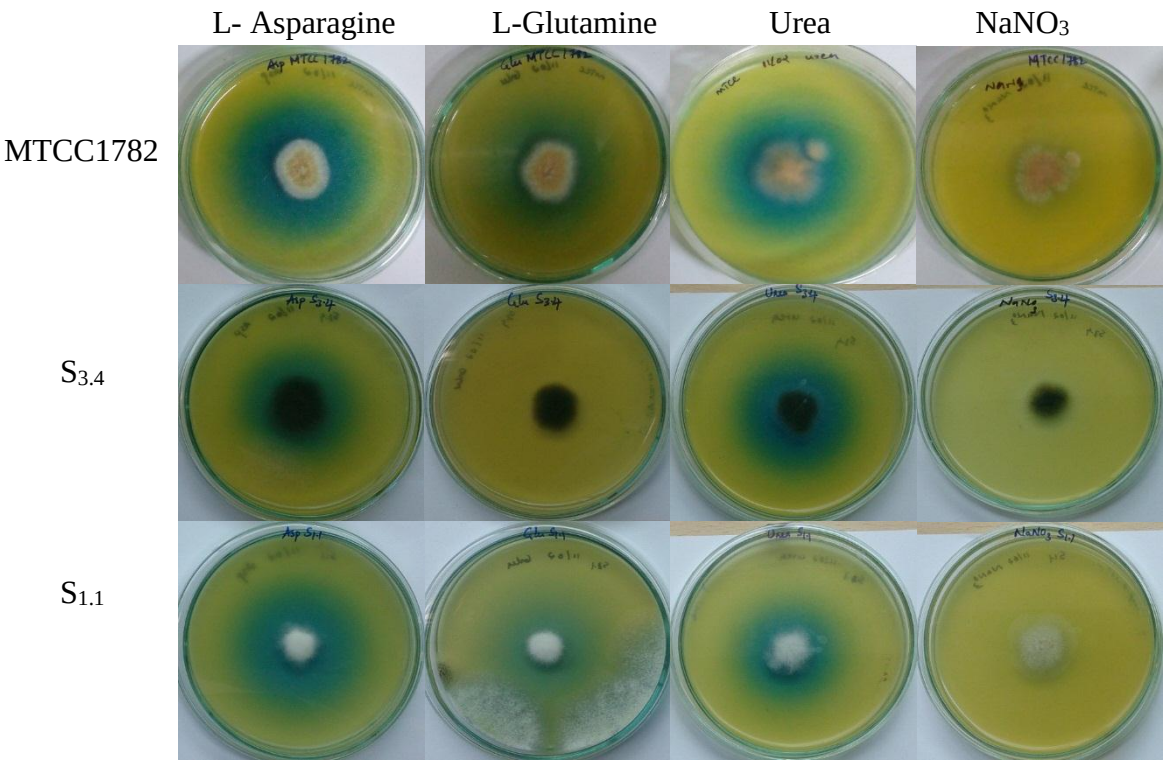
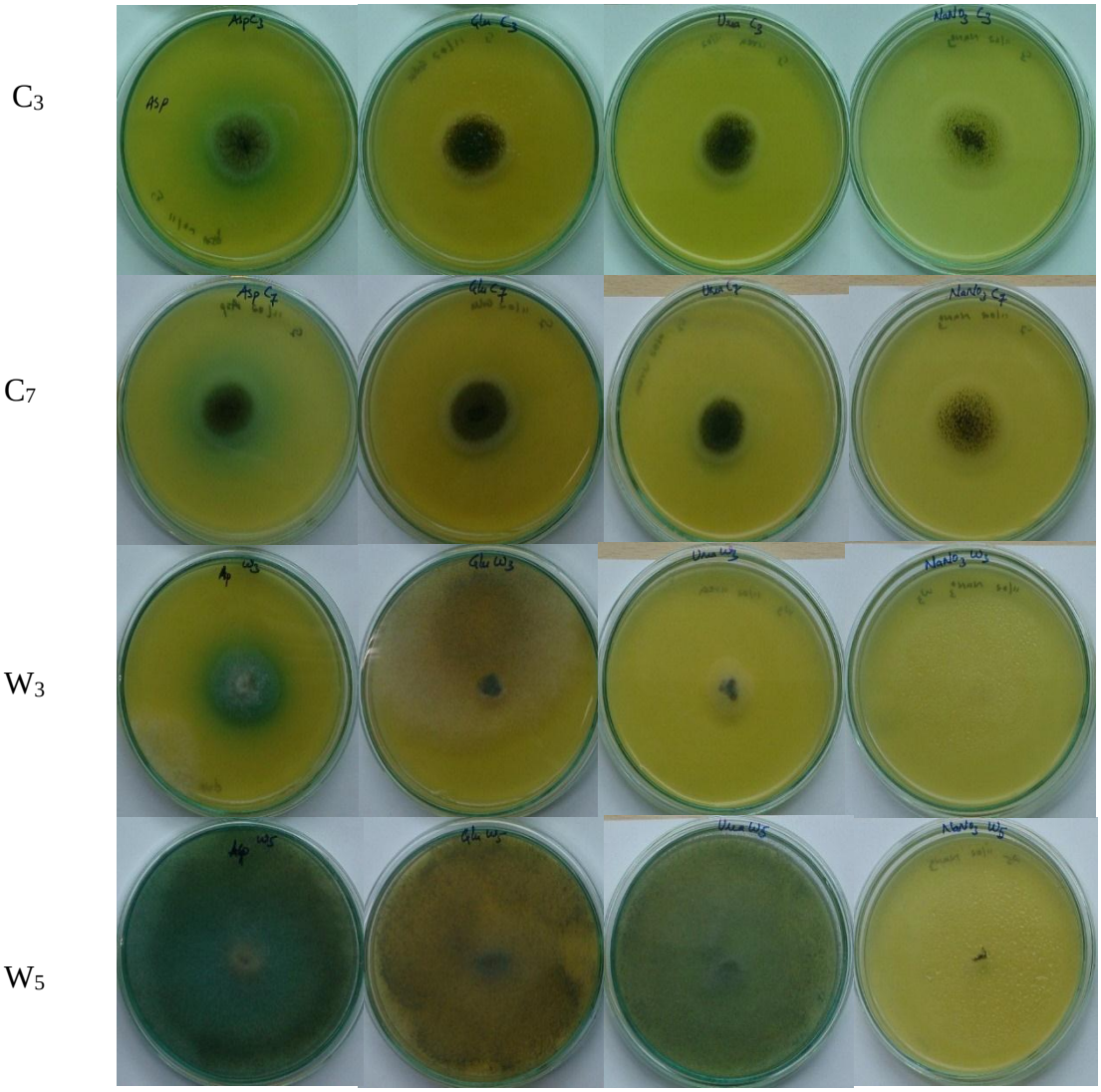


Figure 5. Screening of isolated strains for multiple enzyme activity using MCD plates amended with .007% BTB





4.3 Semi quantitative studies of the isolated fungal strains

Enzyme activity can be calculated semi quantitatively by calculating the relative ratio of zone diameter to colony diameter called zone index. The comparison of zone index values of isolates $S_{3.4}$, W_3, W_5, C_3, C_7 and MTCC 1782 *Aspergillus terreus* strain using phenol red and BTB dye are given in Table 8. Measurement of zone diameter and colony diameter is depicted in figure 6. Using this qualitative plate assay rapid screening of the fungi for the synthesis of the enzyme by direct visualization and activity of the enzyme can be measured.

Gulati et al., revealed that equivalent relation exists between zone index and enzyme activity measured from broth. In the current work enzyme index varied from 0.8 to 4, which is in line with study conducted by Abhinav et al. 2010 [94]. Isolated fungi W_3, W_5, C_3, C_7 and $S_{3.4}$ were cultured in PDA slants, later morphologically identified as *Culvularia* sp., *Rhizopus* sp., and *Aspergillus* sp., respectively [95]. Microscopic pictures of these strains are shown below.

Figure 6. Picture showing zone diameter and colony diameter

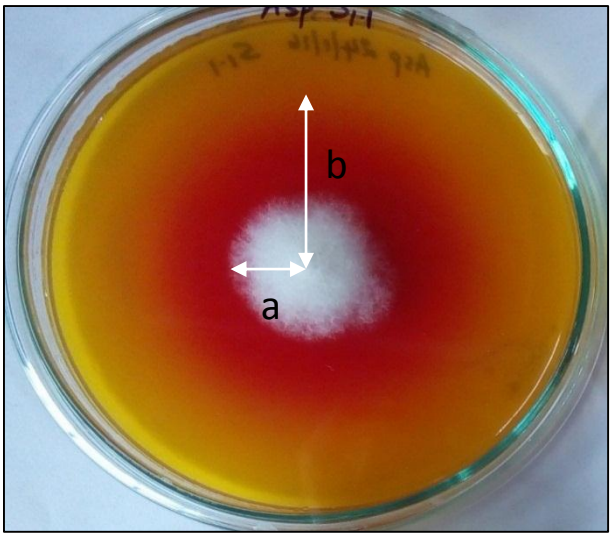


Table 7: Fungal species screened for multi enzyme production

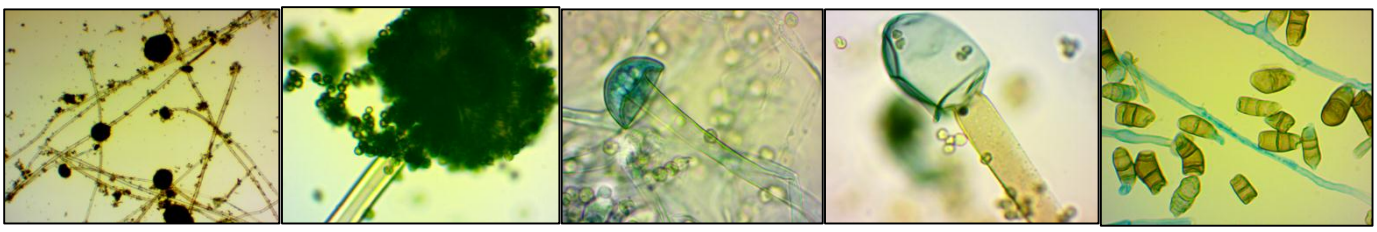
Isolation source	Isolate	Control ($NaNO_3$)	Urea	L-asparagine	L-glutamine	L-Asparaginase enzyme index		
						Colony diameter (cm)	Zone Diameter (cm)	Zone index
Soil from Western Ghats	$S_{1.1}$	-	+	+	+	2	6.5	3.25
	$S_{1.4}$	-	+	+	+	2.1	3.9	1.86
	$S_{2.1}$	-	+	+	+	3.2	8.5	2.66
	$S_{3.4}$	-	+	+	-	3.3	6.8	2.06
	$S_{4.1}$	-	+	+	+	2	8.5	4.25
Red gram husk	P_2	-	+	+	+	3.5	5.5	1.57
	P_3	-	+	+	+	2.5	3	1.20
Rice husk	R_1	-	+	+	+	2.6	6	2.31

	R_3	-	+	+	+	3.7	6.3	1.70
	W_1	-	+	+	-	6	6.5	1.08
	W_2	-	+	+	+	4	7	1.75
Wheat bran	**W_3**	**-**	**-**	**+**	**-**	**3.7**	**4.7**	**1.27**
	W_4	-	+	+	+	2	4	2.00
	W_5	**-**	**-**	**+**	**-**	**2.2**	**2.6**	**1.18**
	C_1	-	+	+	+	2.5	6	2.40
	C_3	**-**	**-**	**+**	**-**	**3.8**	**5.5**	**1.45**
Cotton seed oil cake	C_4	-	+	+	-	7.5	7	0.93
	C_5	-	+	+	-	7.5	7	0.93
	C_6	-	+	+	+	8.5	7	0.82
	C_7	**-**	**-**	**+**	**-**	**3.5**	**5.5**	**1.57**
	MTCC 1782	**-**	**+**	**+**	**+**	***2.5***	***6.0***	***2.4***

Table 8: L-Asparaginase enzyme index measurement using phenol red and Bromothymol blue amended in MCD medium and species observed under Light microscope.

Isolate	Phenol Red			Bromothymol Blue			Species
	Colony diameter (cm)	Zone Diameter (cm)	Zone index	Colony diameter (cm)	Zone Diameter (cm)	Zone index	
W_3	2.40	2.40	1.00	3.70	4.70	1.27	*Rhizopus* sp.
W_5	8.80	8.80	1.00	2.20	2.60	1.18	*Rhizopus* sp.
C_3	3.00	3.00	1.00	3.80	5.50	1.45	*Aspergillus* sp.
C_7	3.00	4.60	1.53	3.50	5.50	1.57	*Aspergillus* sp.
$S_{3.4}$	4.30	6.70	1.56	3.30	6.80	2.06	*Curvularia* sp.
MTCC1782	2.50	6.00	2.40	2.50	6.00	2.40	*Aspergillus* sp.

Figure 7. Microscopic pictures of isolated strains



a b c d e

a - C_3: *Aspergillus* sp.

b - C_7: *Aspergillus* sp.

c - W_3: *Rhizopus* sp.

d - W_5: *Rhizopus* sp.

e - $S_{3.4}$: *Curvularia* sp.

4.4 Quantitative studies of L-Asparaginase activity

The L-Asparaginase activity of the four isolated strains with no glutaminase and urease activity are measured in liquid broth studies along with MTCC 1782 strain using the protocol of Sanjay et al (2011) as described in section 3.8.1. The observed values are plotted in fig 8. MTCC strain is found to have the highest activity at 72 h. Among the four isolated strains C_7 has highest activity. All the strains exhibit the highest activity at 72 h.

Figure 8. Activity plots of isolated fungal strains

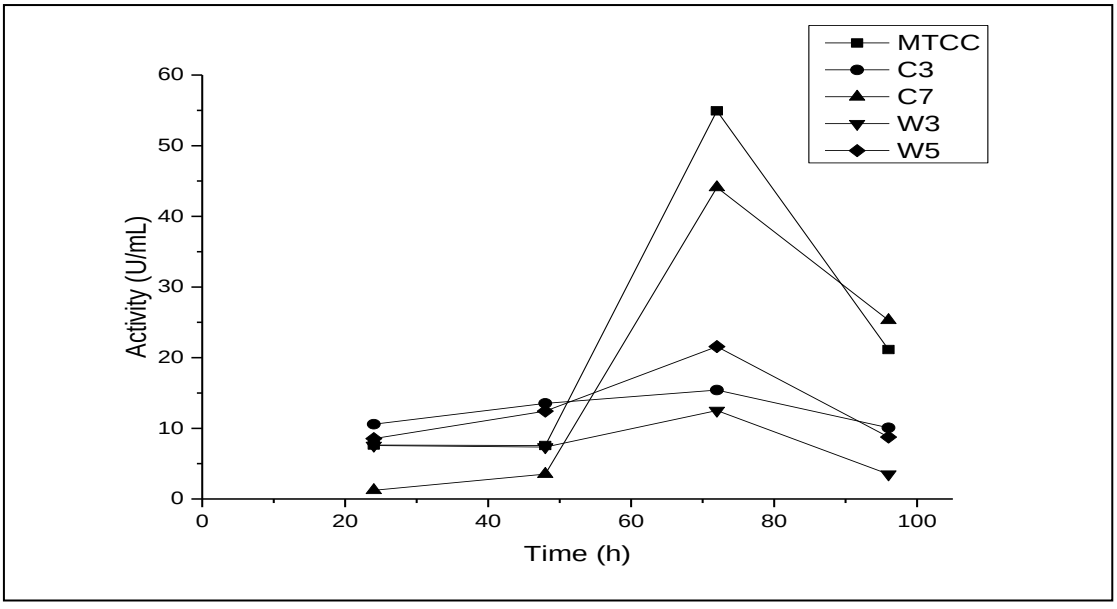


Table 9. Activity values of isolated fungal strains

	Time h	24	48	72	96
Activity (U/mL)	MTCC	7.63	7.57	54.92	21.15
	C_3	10.57	13.52	15.42	10.07
	C_7	1.22	3.52	44.09	25.29
	W_3	7.57	7.35	12.51	3.51
	W_5	8.54	12.43	21.57	8.77

4.5 Protein estimation studies

Protein values of the 4 isolated strains are calculated with Lowry's method as described in section 3.8.2. The specific activity values of the fungi are obtained by dividing the activity values with corresponding protein content values.

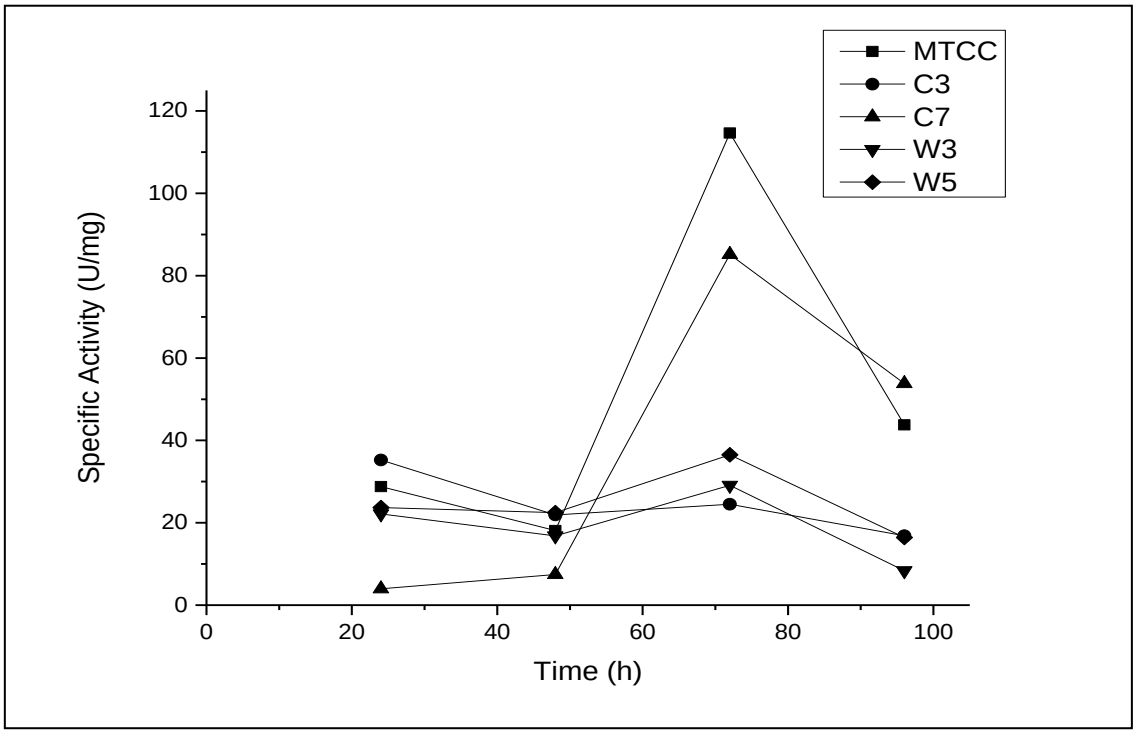
Table 10. Protein content values of isolated strains.

	Time h	24	48	72	96
Protein content mg/ml	MTCC	0.265	0.418	0.479	0.483
	C_3	0.300	0.617	0.629	0.598
	C_7	0.309	0.474	0.518	0.470
	W_3	0.342	0.437	0.430	0.419
	W_5	0.361	0.553	0.591	0.534

Table 11. Specific activity values of isolated strains.

	Time h	24	48	72	96
Specific activity U/mg	MTCC	28.80	18.10	114.64	43.77
	C_3	35.25	21.92	24.51	16.83
	C_7	3.96	7.43	85.17	53.80
	W_3	22.15	16.81	29.08	8.36
	W_5	23.69	22.48	36.50	16.43

Figure 9. Specific activity plots for isolated strains.



4.6 L-Asparaginase activity studies in SSF

The solid state fermentation studies of two strains C_7 and W_5 were conducted in an Erlenmeyer flask with 20 g wheat bran as the substrate. The moisture content was adjusted to 60% with pH 7 sodium phosphate buffer and incubated at 30°C. The following results are observed. The SmF is found to have more activity compared to SSF. And C_7 has more activity than W_5 in SSF also.

Table 12. Comparison of SSF and SmF activity values for W_5

W_5		
time h	SSF activity U/ml	SMF activity U/ml
24	4.76	8.54
48	6.21	12.43
72	15.28	21.57
96	8.45	8.77

Figure 10. Comparison of SSF and SmF activity values for W_5

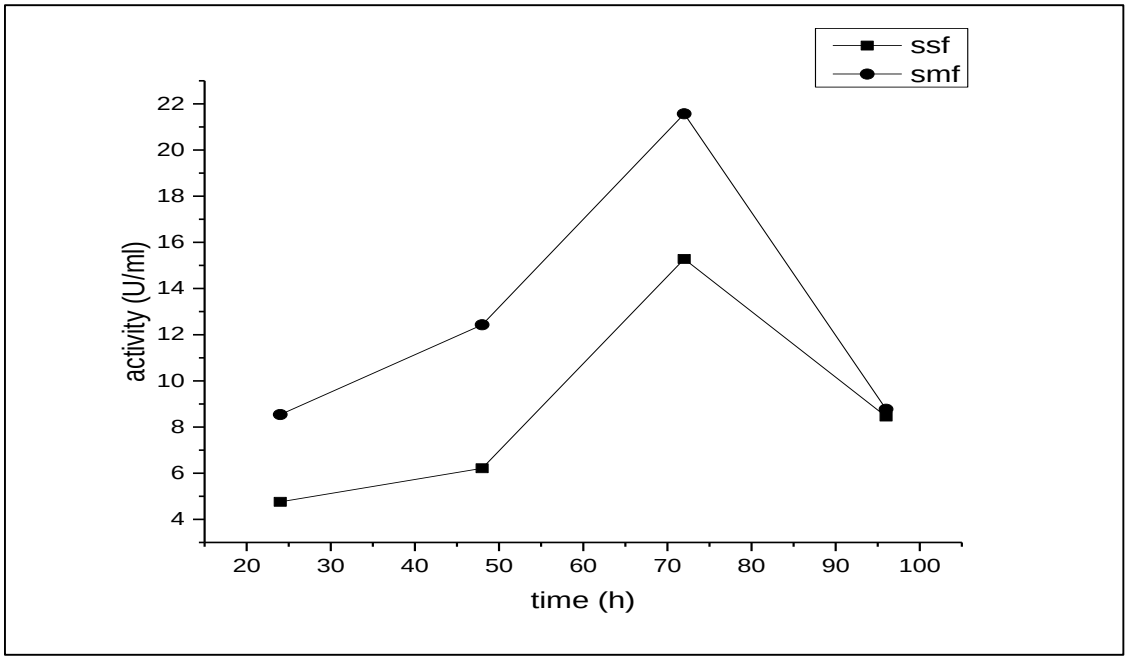
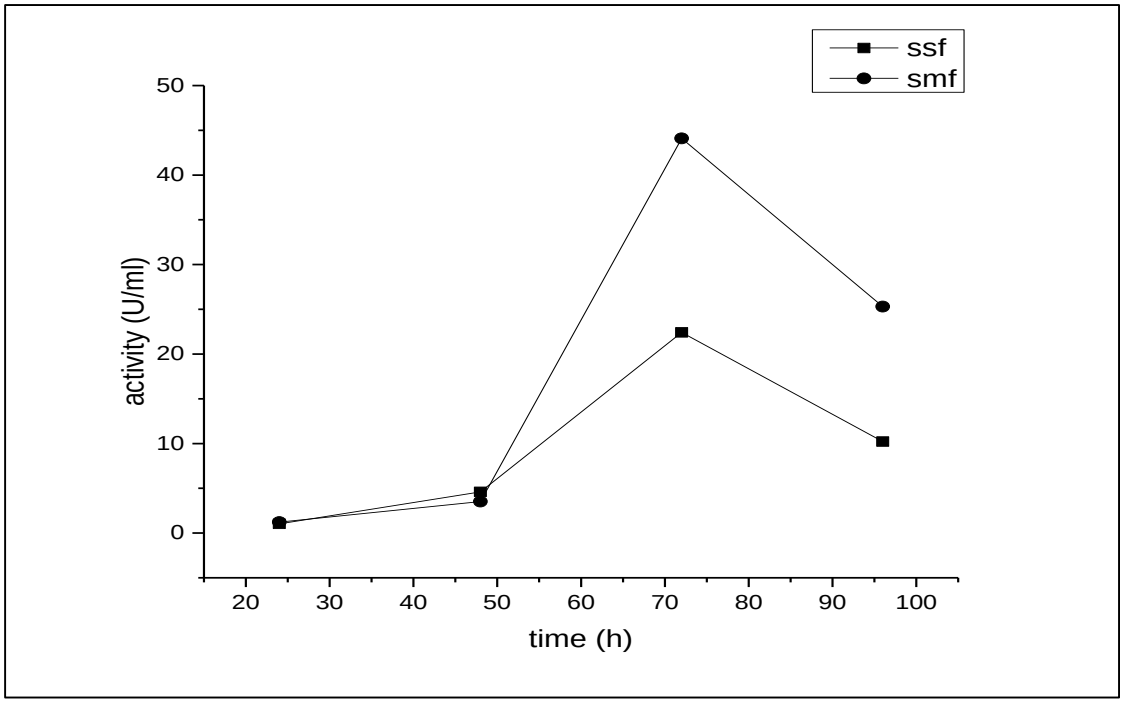


Table 13. Comparison of SSF and SmF activity values for C_7

C_7		
time h	SMF activity U/ml	SSF activity U/ml
24	1.22	1.03
48	3.52	4.6
72	44.09	22.41
96	25.29	10.22

Figure 11. Comparison of SSF and SmF activity values for C_7



Chapter 5

CONCLUSION AND FUTURE STUDIES

In conclusion, L-Asparaginase from various microbial sources exhibits properties that acts as an anti-neoplastic agent and reduces acrylamide levels in food. Even though Bacterial L-Asparaginase is clinically applied for treatment of ALL, it induces adverse reactions. For food and pharmaceutical industry applications, further studies to extract L-Asparaginase from novel sources such as eukaryotic organisms and marine sources should be explored such that extracted enzyme has high specificity towards L-asparagine and prolonged half-life.

In the present study, 4 fungal strains producing glutaminase and urease free L-Asparaginase was screened and isolated so as to reduce the multiple enzyme activity thereby reducing toxic effects. Efforts are made to isolate L-Asparaginase from various microbial sources and understand the mechanism of L-Asparaginase sensitivity to improve efficacy of the enzyme. The strain C_7 is found to have the highest activity out of the four strains without any glutaminase and urease activity and the activity values are 44.09 U/ml and 22.41 U/ml in SmF and SSF respectively.

Further studies should be carried out in the following areas.

- Optimization of the various factors which are affecting the production of the enzyme including temperature, pH, media components, inoculum concentration, rpm etc.
- Statistical design methods can be used for the optimization of these factors which influence the activity.

- Scale up of the enzyme production to semi pilot and pilot plant levels.
- Downstream processing of the isolated enzyme.
- Cytotoxic and immunogenic properties of the isolated enzyme.

Chapter 6

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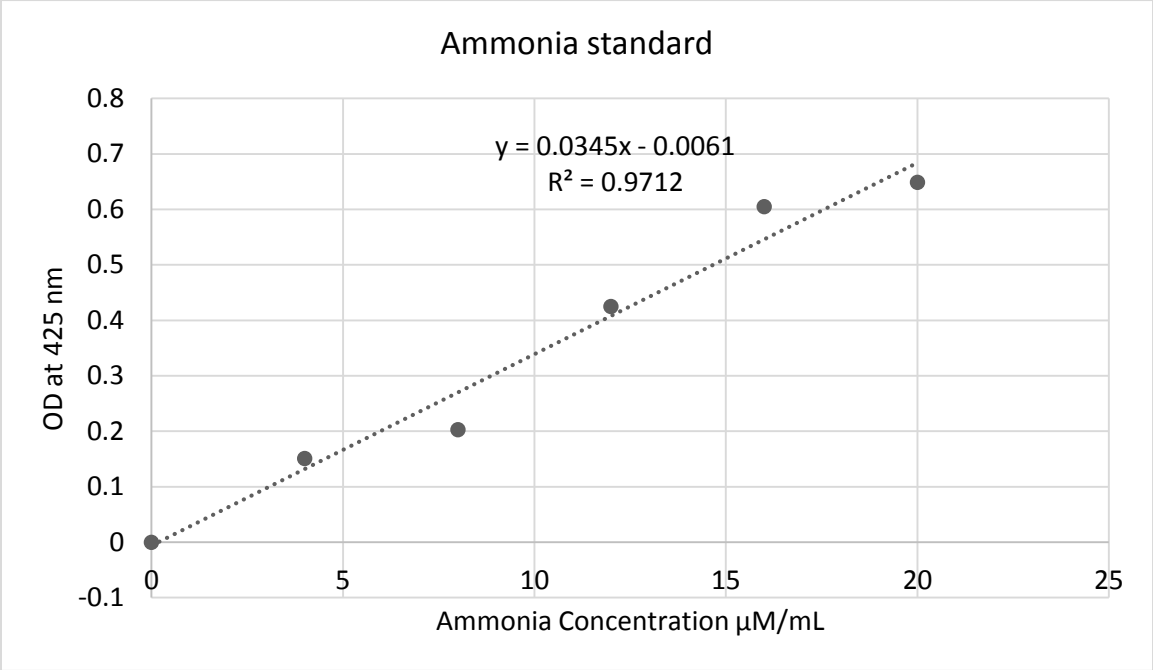
APPENDIX

A.1 Calculation of L-Asparaginase activity

A stock solution of ammonium sulfate with concentration 1 µm/ml was prepared in 50 mM tris buffer of pH 8. It is diluted with milliQ water to get different concentrations viz .2 to 1. Ammonium sulfate is a divalent alt of ammonia hence 1 mol of ammonium sulfate release 2 mol of ammonia. Hence 1 µm/ml solution of ammonium sulfate is equivalent to 2 µm/ml of ammonia. Experiments were conducted in triplicates for the standard plot to observe the absorbance at 425 nm against appropriate blanks as described in the protocol. The obtained points are plotted in Microsoft Excel.

Figure A.1. Ammonium sulfate standard curve for ammonia



L-Asparaginase activity is calculated from the following equation.

Activity (units/mL of enzyme) = (µ mole of NH_3 liberated* V_1)/(V_2*t*V_3)

V_1- Volume of test sample in step 1, V_2- volume of step 1 supernatant used in step 2, V_3- Volume of crude enzyme used in step 1, t- time of incubation.

One unit of L-Asparaginase (U) is defined as the amount of enzyme which liberates 1µmol of ammonia per min at 37 °C.

A.2 Calculation of protein values

A stock solution of .1 mg/ml of bovine serum albumin is prepared in milliQ water. It was diluted to get standard solutions of various concentrations from 0.01 to 0.1 mg/mL. Experiments were done for standard curve in triplicates for the standard values and absorbance is measured at 660 nm against appropriate blank as described in protocol. The obtained points are plotted in Microsoft Excel.

Figure A.2. Standard curve of bovine serum albumin.